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Sharp-1/DEC2 Inhibits Skeletal Muscle Differentiation through Repression of Myogenic Transcription Factors*

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Skeletal muscle differentiation is regulated by the basic-helix-loop-helix (bHLH) family of transcription factors. The myogenic bHLH factors form heterodimers with the ubiquitously expressed bHLH E-proteins and bind E-box (CANNTG) sites present in the promoters of several muscle-specific genes. Our previous studies have shown that the bHLH factor Sharp-1 is expressed in skeletal muscle and interacts with MyoD and E-proteins. However, its role in regulation of myogenic differentiation remains unknown. We report here that endogenous Sharp-1 is expressed in proliferating C2C12 myoblasts and is down-regulated during myogenic differentiation. Constitutive expression of Sharp-1 in C2C12 myoblasts promotes cell cycle exit causing a decrease in cyclin D1 expression but blocks terminal differentiation. Although MyoD expression is not inhibited, the induction of differentiation-specific genes such as myogenin, MEF2C, and myosin heavy chain is impaired by Sharp-1 overexpression. We demonstrate that the interaction of Sharp-1 with MyoD and E-proteins results in reduced DNA binding and transactivation from MyoD-dependent E-box sites. Re-expression of MyoD~E47 rescues the differentiation defect imposed by Sharp-1, suggesting that myogenic bHLH factors function downstream of Sharp-1. Our data suggest that protein-protein interactions between Sharp-1, MyoD, and E47 resulting in interference with MyoD function underlies Sharp-1-mediated repression of myogenic differentiation.

Members of the basic helix-loop-helix (bHLH)[1] family of transcription factors regulate differentiation of many cell types, including neural, skeletal muscle, and hematopoietic cells (1–3). The bHLH family has recently been classified into six phylogenetic groups A–F, which is based on sequence comparisons, DNA binding sites, as well as presence of conserved residues and specific domains (4, 5). The class A group includes factors such as MyoD and E-proteins, which bind to the E-box sequences CACCTG/CAGCTG. Class B includes the ubiquitously expressed Myc family, which also binds the E-box sequences CACGTG/CATGTG. The class C subfamily includes the bHLH-PAS proteins Sim1, HIF, and Clock that have an additional PAS domain and bind the sequence ACGTG/GCGTG. Class D is represented by Id proteins that lack a basic domain and therefore do not bind DNA and function as antagonists of class A proteins. Class E includes the Hairy and Enhancer of Split [E(Spl)] subfamily, which preferentially binds the N-box sequence CACGCG and is characterized by an orange domain as well as a tetrapeptide motif WRPW. Class F corresponds to the Coe family, which has an additional COE domain involved in dimerization and DNA binding.

Skeletal muscle differentiation is regulated mainly by two families of transcription factors: the bHLH myogenic regulatory factors (MRFs), which include MyoD, Myogenin, Myf5, and MRF4, and myocyte enhancer factor 2 (MEF2) factors, which belong to the MADS box family. All MRFs contain a conserved basic domain for DNA binding, and the HLH domain, which is required for heterodimerization with the ubiquitously expressed bHLH E-proteins E12, E47, or HEB (6). Heterodimers of MRFs and E-proteins bind to E-box sequences (CANNTG) present in the promoters of muscle-specific genes (7, 8). The four vertebrate MEF2 family members, MEF2A–D, form homodimers or heterodimers and bind to AT-rich MEF2 binding sites (9), which are also found in all muscle-specific genes. In addition to forming an auto- and cross-regulatory transcriptional loop, MRFs and MEF2 transcription factors physically interact and synergistically activate skeletal muscle specific promoters (9, 10).

Although all four myogenic bHLH factors are capable of inducing myogenic differentiation when expressed in a variety of non-muscle cells (11, 12), gene disruption studies have indicated that MyoD and Myf5 are required for myogenic determination, whereas myogenin and MRF4 play a role in terminal differentiation (13–17). Skeletal muscle differentiation has been widely studied in the myoblast cell line C2C12. Differentiation of C2C12 myoblasts is a multistep process that requires a coordinated sequence of molecular events, involving an initial withdrawal of cells from the cell cycle, followed by expression of terminal differentiation genes and subsequent fusion of cells into multinuclear myotubes. Consequently, regulators of cell cycle progression as well as terminal differentiation have been found to modulate myogenic differentiation. For instance, protooncogenes such as Ras, c-Jun, Fos, and cyclin D1 prevent cell cycle exit and inhibit differentiation of muscle cells (18–20). Conversely, the cyclin-dependent kinase inhibitors p21 and p57 are positively required for myogenic differentiation (21). Several regulators of differentiation genes have also been shown to inhibit myogenesis, which include Notch (22–24) and TGFβ (25).

MyoD and Myf5 are expressed in proliferating myoblasts (12, 26, 27), yet activation of muscle differentiation does not occur until cells are depleted of growth factors, which allows for a permanent exit from the cell cycle. The myogenic activity of

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[1] The abbreviations used are: bHLH, basic helix-loop-helix; HDAC, histone deacetylase; E(Spl), Enhancer of Split; RT, reverse transcription; MHC, myosin heavy chain; MCK, muscle creatine kinase; MRF, muscle regulatory factors; GM, growth medium; DM, differentiation medium; TGFβ, transforming growth factorβ; TBS, Tris-buffered saline.

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MyoD in proliferating myoblasts is restrained through a variety of regulators. Although the precise mechanisms by which MyoD activity is inhibited is unclear, negative regulators of MyoD activity such as Id, Twist, Mist, MyoR, and Hes1 have been reported, which regulate MyoD activity through active or passive mechanisms. For instance, Id lacks a DNA binding domain and functions primarily by sequestration of E-proteins (28), whereas Twist inhibits both MyoD and MEF2 activities (29). *In vitro* studies have indicated that MyoR interacts with E-proteins and binds to E-box sites to repress myogenesis (30). On the other hand, Mist1 homodimers bind E-box sites but also inactivate MyoD by forming inactive MyoD-Mist heterodimers (31). In addition to these bHLH regulators, MyoD is associated with HDAC1 in proliferating myoblasts, and this interaction is important for inhibition of MyoD transcriptional activity (32).

We have recently described the cloning and transcriptional properties of the mouse bHLH factor Sharp-1/Dec2 (33), which belongs to the Hairy/[E(Spl)] subfamily of bHLH factors. Sharp-1 and Stra13 share the highest sequence homology with each other relative to the remaining family members (34–37). Both proteins can function as transcriptional repressors but lack a WRPW motif in the C terminus, which characterizes the remaining family members. Moreover, both Sharp-1 and Stra13 have been found to bind to the E box motif (CANNTG) (33, 38). Although Sharp-1 is expressed in a number of tissues (33, 39, 40), and the mouse, rat, and human homologs have been identified (40), no function for this protein has been described. We have shown recently that Sharp-1 is expressed in skeletal muscle (33) and can interact with both MyoD and E-proteins suggesting that it may regulate myogenesis.

In this study, we have examined the role of Sharp-1 in regulation of myoblast differentiation. We demonstrate that endogenous Sharp-1 expression is down-regulated during muscle differentiation and that its constitutive overexpression in C2C12 myoblasts results in inhibition of terminal differentiation. Interestingly, Sharp-1-overexpressing cells exhibit an early exit from the cell cycle relative to control C2C12 cells, indicating that inhibition of muscle differentiation by Sharp-1 occurs independent of effects on cell cycle progression. Sharp-1 inhibits myogenic bHLH transcription factor activity by interaction with myogenic bHLH proteins. Thus, Sharp-1-overexpressing cells have reduced MyoD activity resulting in impaired expression of differentiation specific genes such as p21, myogenin, MEF2C, and MHC. The differentiation defect imposed by Sharp-1 overexpression can be partially rescued by forced re-expression of MyoD~E47 tethered dimers indicating that the myogenic bHLH factors function downstream of Sharp-1. Our results provide evidence that Sharp-1 regulates myogenesis by inhibiting myogenic bHLH factor activity.

EXPERIMENTAL PROCEDURES

Cell Culture and Generation of Stable Cell Lines Overexpressing Sharp-1—C2C12 cells were maintained in growth medium (GM) consisting of Dulbecco's modified Eagle's medium with 20% fetal bovine serum. For differentiation experiments, C2C12 cells were grown to confluence and then transferred to differentiation medium (DM), consisting of Dulbecco's modified Eagle's medium plus 2% horse serum. Differentiation media was replaced every 48 h. To obtain stable transfectants overexpressing Sharp-1, C2C12 cells were co-transfected with a 1:10 ratio of an expression vector for Sharp-1 (pCS2-Sharp-1) and the neomycin resistance plasmid pKJ-1. 48 h after transfection, cells were selected in medium containing 500 μg/ml G418 (Invitrogen). After 14–21 days, G418-resistant clones were isolated, expanded, and screened by RT-PCR and Western blot analyses. The expression levels of Sharp-1 in individual clones was determined by RT-PCR using primers RT18 (5′-TTTCCCTTGCTTGTCGTCCG-3′) and RT8 (5′-AGAGTAAGAGATGCTCTGCT-3′), which amplify a 573-bp fragment, and Western blot analysis using anti-Sharp-1 antibodies (kindly provided by Dr. B. Yan, University of Rhode Island). Two independent clones (2b and 2d) were used for all studies and showed an identical phenotype. C3H10T1/2 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Western Blot Analysis—Cells were lysed in radioimmune precipitation assay buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 0.5 mm dithiothreitol, and 0.1 mM EDTA) supplemented with protease inhibitor mixture (Roche Applied Science). Equal amounts of extracted proteins (80 μg) were loaded, separated by 9% or 12% SDS-polyacrylamide gels, and transferred at 100 V for 1 h onto nitrocellulose membranes. Membranes were blocked for 2 h in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 5% skimmed milk and then incubated overnight at 4 °C with primary antibodies diluted in blocking solution. The following primary antibodies were used at a dilution of 1:500: MyoD, Myogenin, p21, Cyclin D1, E2A.E12 (Santa Cruz Biotechnology) and EF-1α (Upstate Biotechnology). After incubation with primary antibody, membranes were washed in TBST (TBS with 0.1% Tween 20) containing 1% milk and then incubated with secondary antibody conjugated to HRP (Sigma) for 1 h. After three washes with the above solution and one wash with TBST, proteins were detected using ECL reagents.

RT-PCR Analysis—Total RNA was isolated from C2C12 and C2C12-Sharp-1 cells with TRIzol (Invitrogen). The amounts of RNA were normalized to the ubiquitous 36B4 transcripts as described previously (33). To detect endogenous Sharp-1 upon differentiation of C2C12 cells, primers RT8 and RT9 (33) were used, which amplified a 280-bp fragment. RT-PCR products were detected by Southern blot analysis with ^{32}P-labeled Sharp-1 and 36B4 cDNA probes. The primers used for amplification of MEF2C (RT208 and RT209) are as follows: 5′-GTATGTCTCCTGGTGTAACA-3′ and 5′-GGATATCCTCCCATTCCTTG-3′.

Immunohistochemistry—Immunohistochemistry was performed as described (33). Briefly, to detect myosin heavy chain (MHC) expression, C2C12 cells cultured in DM for 3 days were fixed in methanol for 20 min at 4 °C. Following a 30-min blocking step (5% goat serum in phosphate-buffered saline), plates were incubated with 1:400 dilution of MY-32, a monoclonal anti-skeletal myosin antibody (Sigma) specific for the myosin heavy chain, overnight at 4 °C. The primary complexes were detected using a biotinylated anti-mouse antibody (Vector Laboratories, Inc., Burlingame, CA), and a horseradish peroxidase-streptavidin conjugate (Vector Laboratories). Specific immunocomplexes were visualized by 3,3′-diaminobenzidine tetrahydrochloride (Fluka), resulting in brown immunoreactive sites.

Bromodeoxyuridine Staining—Bromodeoxyuridine (BrdUrd) was added to the media of growing cells at a concentration of 10 μM for 2–3 h. Cells were washed with phosphate-buffered saline and fixed with methanol for 20 min, and the endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min at 37 °C. After a phosphate-buffered saline wash, cells were incubated with 2 N HCl solution for 30 min. This was again followed by a phosphate-buffered saline wash, and subsequently the cells were incubated with 1:500 monoclonal anti BrdUrd antibody at 4 °C overnight. The remaining procedure was similar to immunostaining of cells as described above.

Rescue/Differentiation Assay—C2C12-Sharp-1 cells were transfected with expression vectors for MyoD, MyoD~E47 tethered construct, or an empty vector. Parental C2C12 cells were also transfected with empty vector as control. After 24 h, cells were switched to DM, and the culture was continued in the medium for 3 days. Differentiation was quantified by counting the number of multinucleated myotubes in at least four different fields. Cells were lysed with radioimmune precipitation assay buffer for Western blot analysis or fixed with ice cold methanol for immunostaining.

Luciferase Assays—5×10^4 cells were plated 1 day before transfection in 24-well plates and transiently transfected with plasmids as indicated using LipofectAMINE Plus (Invitrogen). Empty expression vectors were added to normalize the amount of DNA in each well. Cells were harvested with passive lysis buffer, and luciferase assays were performed according to the manufacturer's instructions (Promega, Madison, WI). Transfection efficiencies were normalized by co-transfecting 50 ng of the β-galactosidase plasmid pCH110 (Amersham Biosciences). All transfections were performed in duplicate at least three times.

Gel Shift Assays—Sharp-1, E47, MyoD, and MyoD~E47 tethered proteins were translated *in vitro* using the TNT-coupled transcription/translation system (Promega), and the efficiency of translation was monitored by Western blot analysis. Gel shift assays were performed using end-labeled double-stranded oligonucleotides corresponding to the E box site from the MCK enhancer (5′-GATCCAACACCTGCTGCCTGAG-3′), and one containing multimerized class A E-box sites (5′-GGCCGCAGCAGCTGGCACAGCAGCTGGCACAGCAGCTGGCAGC-3′). Binding reactions contained 40,000 cpm probe, 1 μg of poly(dI-dC),

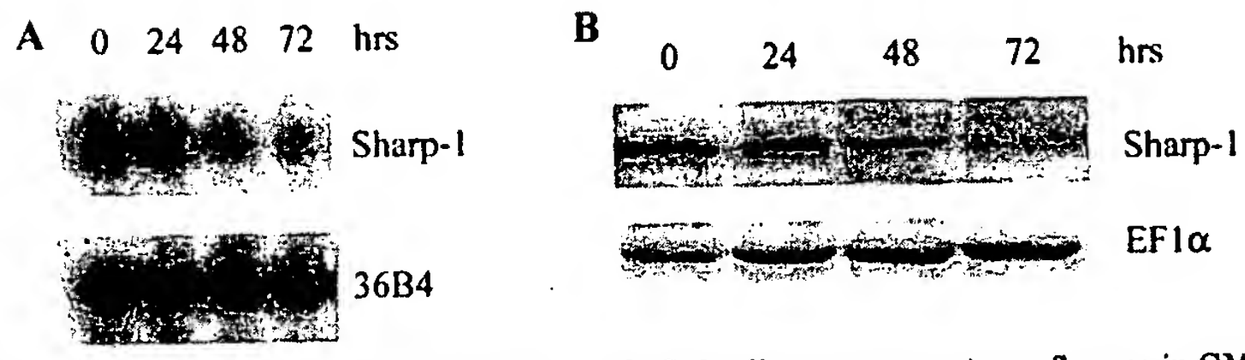


FIG. 1. **Expression of Sharp-1 during myoblast differentiation.** C2C12 cells were grown to confluence in GM (0 h) and then shifted to DM for a period of 24–72 h. *A*, samples were harvested at various time points and analyzed for Sharp-1 transcripts by semi-quantitative RT-PCR. The amounts of RNA were normalized with 36B4 transcripts, which served as an internal control. *B*, Western blot analysis of Sharp-1 expression on the same samples. In each *lane*, equal loading was assessed with anti-EF1α antibody.

10 mM HEPES, pH 7.9, 80 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 10% glycerol, and varying amounts of *in vitro* translated proteins. The reaction was incubated at room temperature for 20 min and fractionated on 5% polyacrylamide gels. Gels were dried and exposed to x-ray film.

Co-immunoprecipitation Assays—To examine interaction with MyoD, E47, and MyoD~E47, epitope-tagged myc-Sharp-1 was used. C3H10T1/2 cells were transfected with 4 μg of each expression vector individually or together as indicated in the figures. Cells were harvested 48 h post transfection and lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol). The lysates were immunoprecipitated overnight at 4 °C with 2 μl of c-myc mouse monoclonal antibody (9E10). A 50% slurry (25 μl) of protein-A-agarose beads was subsequently added to each sample, and the sample was incubated for an additional 1 h at 4 °C. After being rinsed with lysis buffer, the final bead pellet was resuspended in SDS loading buffer and subjected to protein gel electrophoresis, followed by transfer to nitrocellulose membrane. Western blot analysis was carried out as described above using MyoD or E47 antibody.

RESULTS

Sharp-1 Expression Is Down-regulated during Muscle Differentiation—Our previous studies have indicated that Sharp-1 interacts with MyoD and E-proteins and inhibits MyoD-induced muscle differentiation in C3H10T1/2 fibroblast cells (33). To examine whether Sharp-1 expression is regulated during myogenesis, we examined the endogenous expression of Sharp-1 in the myoblast cell line C2C12. C2C12 cells cultured in growth medium (0 h) or in differentiation medium for up to 72 h were harvested and analyzed for Sharp-1 expression by RT-PCR and Western blotting. Sharp-1 mRNA (Fig. 1*A*) as well as protein (Fig. 1*B*) was expressed in proliferating myoblasts cells cultured in growth medium. However, when these cells were induced to differentiate by transfer to differentiation medium, Sharp-1 transcripts as well as protein were gradually down-regulated after 48 and 72 h. The down-regulation of Sharp-1 expression during C2C12 differentiation suggested that it may play a role in myogenesis.

Constitutive Overexpression of Sharp-1 Inhibits C2C12 Myoblast Differentiation—To investigate the potential role of Sharp-1 in myogenic differentiation, we stably transfected C2C12 cells with an expression vector for Sharp-1. Cells were drug-selected, and several independent colonies were picked and analyzed for the ability to differentiate into myotubes. When placed in differentiation medium containing low serum, control C2C12 cells ceased to proliferate and within 3 days fused to form multinucleated fibers (Fig. 2*A*). In contrast, several Sharp-1-transfected clones (C2C12-Sharp-1) that were similarly analyzed (Fig. 2*A*), showed an almost complete block of myotube formation suggesting that overexpression of Sharp-1 inhibits morphological differentiation of C2C12 myoblasts. We examined the level of Sharp-1 overexpression in clone #2b by semi-quantitative RT-PCR (Fig. 2*B*) and by Western blot analysis (Fig. 2*C*). The exogenously transfected Sharp-1 was expressed at ~4-fold higher levels than the endogenous gene.

To determine the mechanism by which Sharp-1 blocks myogenic differentiation, control C2C12 cells and C2C12-Sharp-1 cells were induced to differentiate in DM and analyzed for muscle differentiation markers by immunostaining and by Western blot analysis. Consistent with a defect in morphological differentiation, the expression of the terminal differentiation marker myosin heavy chain (MHC) was significantly lower in C2C12-Sharp-1#2b cells as assessed by immunostaining (Fig. 3*A*). Moreover, although MyoD expression was not reduced (Fig. 3*B*), the expression of myogenin and MEF2C, which are induced upon differentiation, was impaired in C2C12-Sharp-1 cells compared with control cells (Fig. 3, *B* and *C*). Similar results were obtained with another independent clone ((#2d), Fig. 2, and data not shown) indicating that the differentiation defective phenotype is not a clonal artifact. These results indicate that Sharp-1 inhibits myogenic differentiation at a step downstream of MyoD expression.

Sharp-1 Does Not Delay Cell Cycle Exit of Myoblasts—The inability of the C2C12-Sharp-1 cells to differentiate could be due to defects in cell cycle progression or due to defects in terminal differentiation. To examine cell proliferation under growth and differentiation conditions, we performed BrdUrd incorporation assays. C2C12 cells and C2C12-Sharp-1 cells cultured in GM or in DM for 24 and 48 h were pulsed with BrdUrd for 2 h followed by immunostaining with anti-BrdUrd antibodies. The percentage of S-phase cells was calculated by counting the number of $BrdUrd^+$ nuclei in four different fields relative to the total number of nuclei in each field. C2C12 cells cultured in growth medium had 75% cells in S-phase, whereas only 40% of C2C12-Sharp-1 cells were labeled with BrdUrd (Fig. 4*A*). Following transfer of cells to DM, as expected, the number of BrdUrd-positive cells was reduced in control C2C12 cells after 24 h (50%) and 48 h (30%). However, the number of S-phase cells was even more strikingly reduced in C2C12-Sharp-1 clones cultured in DM after 24 and 48 h (25 and 8%, respectively).

To further substantiate these findings, we examined the expression of cyclinD1 by Western blotting. Previous studies have shown that cyclin D1 levels decline during differentiation of myoblasts (41). As expected, cyclin D1 levels were high in C2C12 cells cultured in GM (time 0) and declined when cells were shifted to DM. Consistent with the BrdUrd experiments, basal cyclin D1 levels were lower in C2C12-Sharp-1 cells cultured in GM (time 0) and declined to almost undetectable levels within 24 h of induction of differentiation (Fig. 4*B*). These experiments suggest that overexpression of Sharp-1 facilitates withdrawal of cells from the cell cycle, and thus the inhibition of differentiation in C2C12-Sharp-1 clones is unlikely to be due to a delay in the cell cycle exit of myoblasts. Interestingly, the expression of $p21^{Cip1}$, another marker of cell cycle arrest, was induced in control C2C12 cells upon induction of differentiation but was not up-regulated in C2C12-Sharp-1 cells (Fig. 4*B*). $p21^{Cip1}$ expression has previously been reported to be transcriptionally regulated by MyoD (42, 43). Thus, the decreased $p21^{Cip1}$ expression suggested that MyoD activity may be reduced in C2C12-Sharp-1 cells.

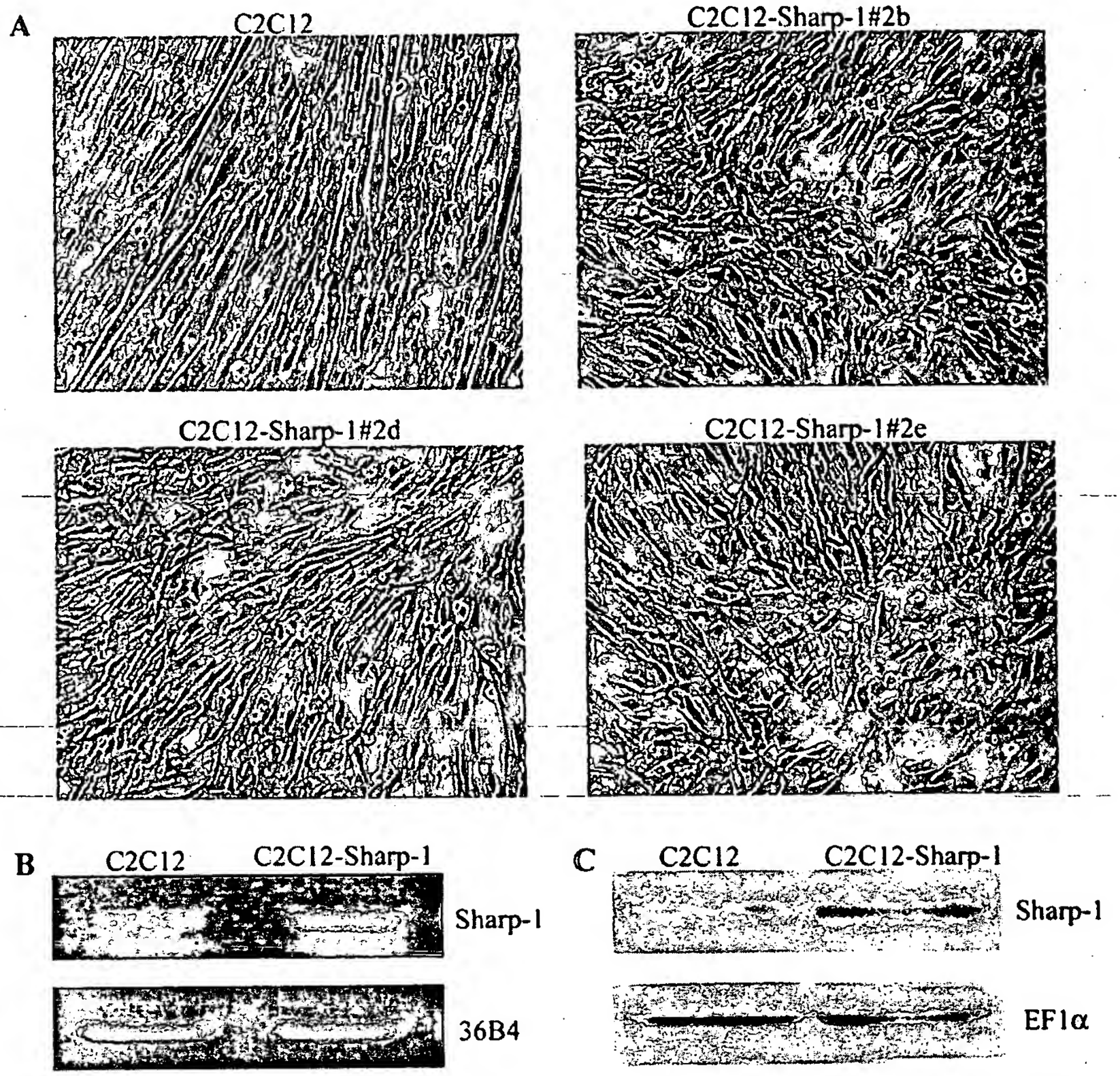


FIG. 2. **Effect of Sharp-1 overexpression in C2C12 cells on myogenesis.** *A*, control C2C12 cells and three independent stable C2C12-Sharp-1 clones (2b, 2d, and 2e) were induced to differentiate in DM for 3 days and analyzed morphologically for myotube formation. *B*, Sharp-1 transcripts in C2C12- and Sharp-1-transfected cells (clone 2b) were analyzed by semi-quantitative RT-PCR. *C*, expression of Sharp-1 protein in control C2C12 and C2C12-Sharp-1 clone 2b was analyzed by Western blot analysis.

Transcriptional Activity of MyoD Is Modulated by Sharp-1—Because MyoD expression is not down-regulated in C2C12-Sharp-1 clones, but myogenic differentiation is, we examined the possibility that MyoD activity is regulated by Sharp-1. To examine MyoD activity, we transfected the MyoD-dependent reporter 4Rtk-luc (44), which contains four tandem E boxes from the muscle creatine kinase (MCK) enhancer upstream of the thymidine kinase basal promoter in control C2C12 and C2C12-Sharp-1 clones. 48 h after transfection, cells were harvested for luciferase activity. Compared with control cells, C2C12-Sharp-1 cells displayed a 50% reduction in 4R-tk-luc activity (Fig. 5*A*) indicating that myogenic bHLH factor activity is inhibited by overexpression of Sharp-1. Furthermore, transfection of MyoD in control C2C12 cells (Fig. 5*B*) resulted in a dose-dependent increase in 4R-tk-luc reporter activity, whereas forced expression of MyoD did not fully rescue reporter activity in C2C12-Sharp-1 cells indicating that the inhibitory effects of Sharp-1 cannot be overcome by MyoD overexpression alone.

Dimerization and DNA-binding Properties of Sharp-1—To identify the mechanisms by which Sharp-1 inhibits MyoD activity, we examined the dimerization and DNA binding properties of Sharp-1. We have previously reported that Sharp-1 interacts *in vitro* with MyoD and with E-proteins (33). To determine whether Sharp-1 complexes with MyoD or E47 *in vivo*, C3H10T1/2 cells were transfected with expression vectors for MyoD or E47 individually and together with myc-Sharp-1. Cell lysates were immunoprecipitated with anti-myc antibody 9E10 and subjected to Western blot analysis with either anti-MyoD or anti-E47 antibodies. Sharp-1 interacted with MyoD (Fig. 6*A*), as well as with E47 (Fig. 6*B*), when co-transfected with either protein, whereas no nonspecific interactions were seen in the negative control lanes where Sharp-1, MyoD, or E47 were transfected individually. These results indicate that Sharp-1 heterodimerizes with MyoD and E47 proteins in cultured cells.

To test the effect of Sharp-1 on MyoD and E-protein binding to E-box sites present in myogenic promoters, we performed electrophoretic mobility shift assays. A ^{32}P-labeled oligonucleotide harboring an E-box site from the MCK promoter (30) was used along with MyoD, Sharp-1, and E47, which were translated *in vitro* using rabbit reticulocyte lysates (Fig. 6*C*). As expected, MyoD and E47 heterodimers exhibited strong binding to the E-box site (*lane 2*), whereas no DNA-protein complex was seen with Sharp-1 alone (*lane 3*), indicating that Sharp-1 does not bind the MCK E-box site as a homodimer or a monomer. MyoD homodimers (*lane 4*) and E47 homodimers (*lane 6*) bound to a lesser degree than MyoD+E47, and formed DNA protein complexes with different mobilities. In the presence of Sharp-1, binding of MyoD homodimers (compare *lane 4* with *5*),

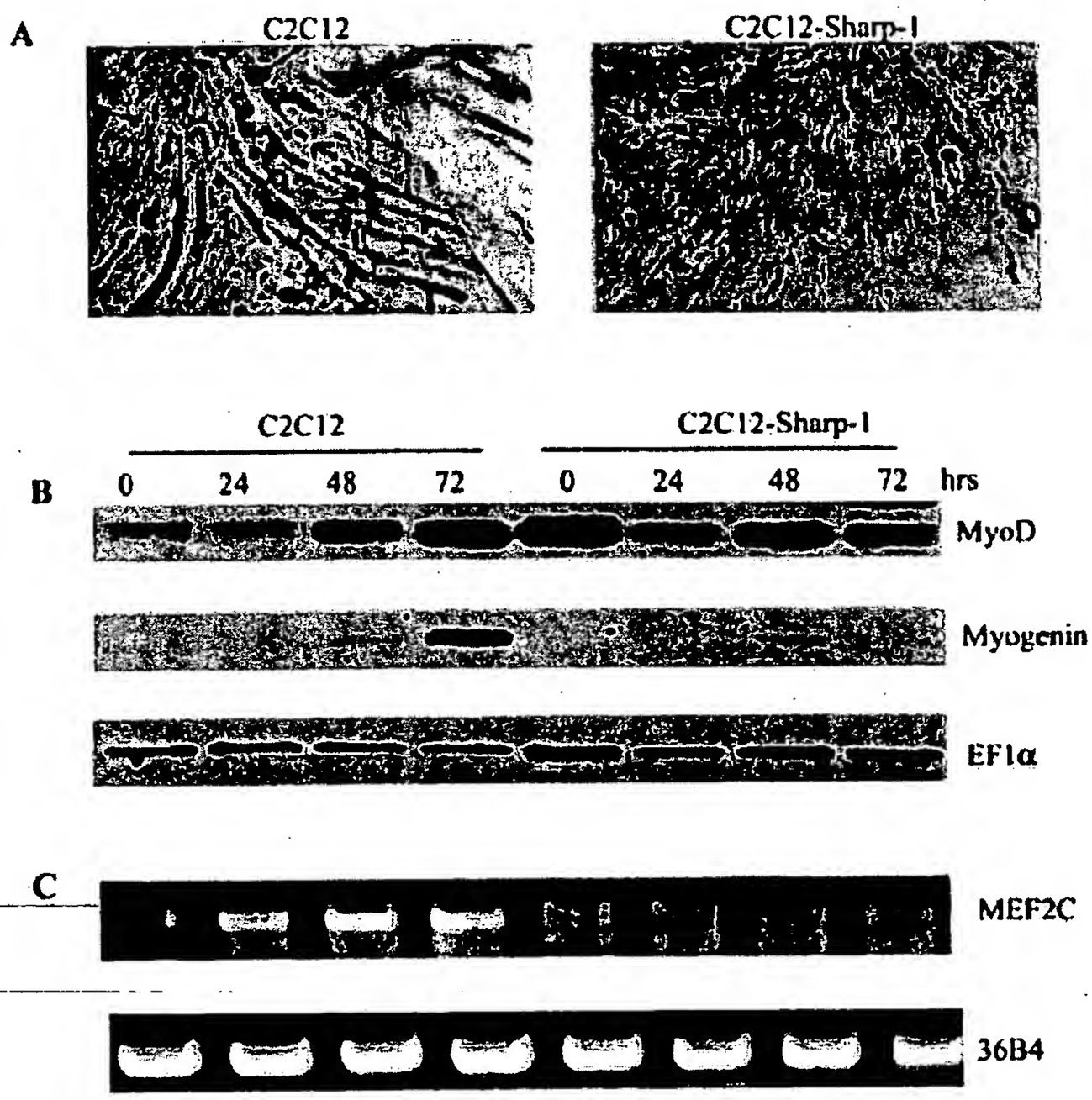


FIG. 3. **Overexpression of Sharp-1 inhibits differentiation of C2C12 cells.** *A*, C2C12 and C2C12-Sharp-1 cells were induced to differentiate in DM for 4 days and assessed for MHC expression by immunostaining with anti-MHC antibody. *B*, expression of MyoD and Myogenin was analyzed in C2C12 and C2C12-Sharp-1 cells cultured in GM and in DM for 24, 48, and 72 h by Western blot analysis. Equal loading was assessed with anti-EF1α antibody. *C*, MEF2C expression was monitored by semi-quantitative RT-PCR in C2C12 and C2C12-Sharp-1 cells. The amount of RNA in each lane was normalized with transcripts to the ribosomal phosphoprotein 36B4.

as well as E47 homodimers (compare *lane 6* with *7*), was reduced. Moreover, addition of Sharp-1 to E47 (*lane 7*) did not result in the formation of a new complex, indicating that Sharp-1 did not bind to this E-box even in presence of E47. These results indicate that, although Sharp-1 itself does not bind to the MCK E-box site either as a homodimer or as a heterodimer with E47, it inhibits MyoD and E47 binding likely by formation of non-DNA binding MyoD-Sharp-1 and E47-Sharp-1 heterodimers. We further tested whether Sharp-1 could inhibit MyoD-E47 heterodimer binding to two different E-box sites using competition gel shift assays (Fig. 6, *D* and *E*). In the absence of Sharp-1, *in vitro* translated MyoD and E47 yielded a DNA-protein complex on the E-box site from the MCK promoter (Fig. 6*D*) as well as on an oligonucleotide containing multimerized class A E-box sites (Fig. 6*E*). Addition of increasing amounts of Sharp-1 effectively reduced DNA binding on both sites, and no additional complexes corresponding to Sharp-1 homodimers or Sharp-1-E47 heterodimers, were detected. Similar results were also obtained with an E-box site derived from the MEF2 promoter (data not shown). Taken together, our studies indicate that, although Sharp-1 does not bind to the E-box sites from muscle promoters that we tested, it prevents MyoD and E-proteins from binding DNA by protein-protein interactions.

Sharp-1 Inhibits MyoD~E47-mediated Activation—To further investigate whether dimerization with MyoD and E-proteins is the primary mechanism by which Sharp-1 inhibits MyoD activity, we examined whether Sharp-1 repressed the activity of a MyoD~E47 tethered dimer, in which MyoD is linked to E47 (45, 46). C3H10T1/2 cells were transfected with 4R-tk-luc reporter along with either MyoD (Fig. 7*A*) or with the tethered MyoD~E47 expression vector (Fig. 7*B*), in the absence or presence of Sharp-1 as indicated. The activity in the presence of Sharp-1 was plotted relative to MyoD or MyoD~E47 alone and given a value of 100%. Co-expression of Sharp-1 repressed MyoD-dependent activation by ~90%, whereas the activity of tethered MyoD~E47 was inhibited by ~50%. The difference in the degree of inhibition of MyoD and MyoD~E47 suggests that Sharp-1 inhibits MyoD activity at least in part by titration of MyoD and E-proteins. Nevertheless, because the MyoD~E47 tethered dimer is not refractory to Sharp-1-mediated inhibition, these results suggest that dimerization with MyoD or E-proteins may not be the only mechanism by which Sharp-1 inhibits myogenesis.

Sharp-1 Inhibits DNA Binding of MyoD~E47 to an E Box Site—Because Sharp-1 partially inhibited the transcriptional activity of MyoD~E47 tethered construct, we first tested whether Sharp-1 interacts with MyoD~E47 heterodimer by co-immunoprecipitation assays. C3H10T1/2 cells were transfected with expression vectors encoding myc-Sharp-1, MyoD~E47 or both. 48 h after transfection, cells were harvested and immunoprecipitated with myc antibody (9E10). The precipitates were run on SDS gels and immunoblotted with MyoD antibody. A strong and specific interaction was detected in cells expressing both Sharp-1 and MyoD~E47 (Fig. 7*C*) demonstrating that Sharp-1 can associate with MyoD even when complexed as a heterodimer with E47.

To examine whether Sharp-1 inhibits the function of MyoD~E47 heterodimer by altering its ability to bind DNA, we performed electrophoretic mobility shift assays using a ^{32}P-labeled oligonucleotide probe containing E-box sites. *In vitro* translated MyoD~E47 and Sharp-1 proteins were made using rabbit reticulocyte lysates. As shown in Fig. 7*D*, *in vitro* translated MyoD~E47 bound strongly to the labeled E box oligonucleotide resulting in a DNA-protein complex. Addition of increasing amounts of Sharp-1 resulted in a dose-dependent decrease in the binding of MyoD~E47 heterodimer indicating that Sharp-1 can titrate this complex off DNA.

Expression of MyoD~E47 Restores Differentiation of C2C12-Sharp-1 Cells—Our experiments suggested that Sharp-1 inhibits

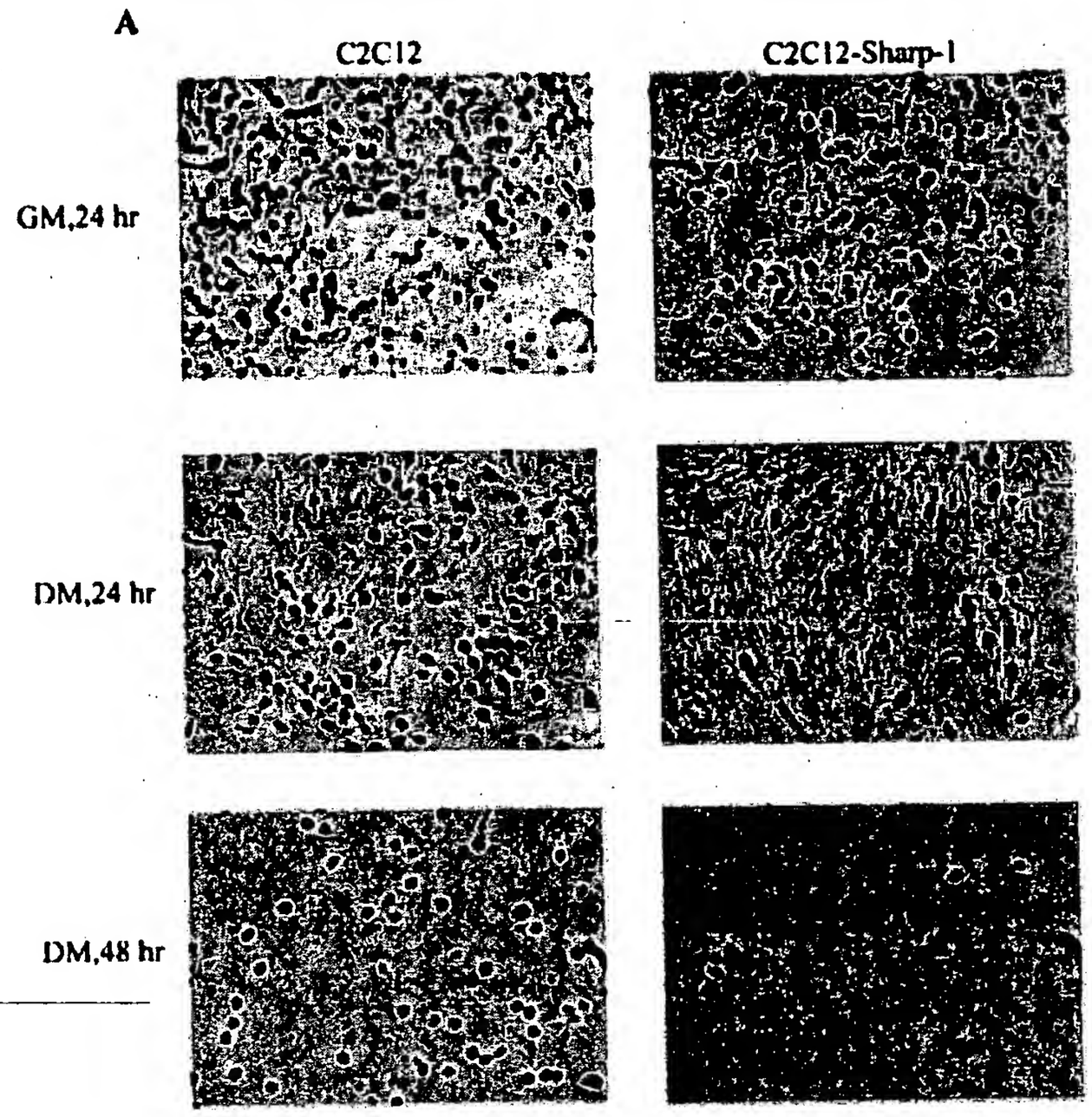


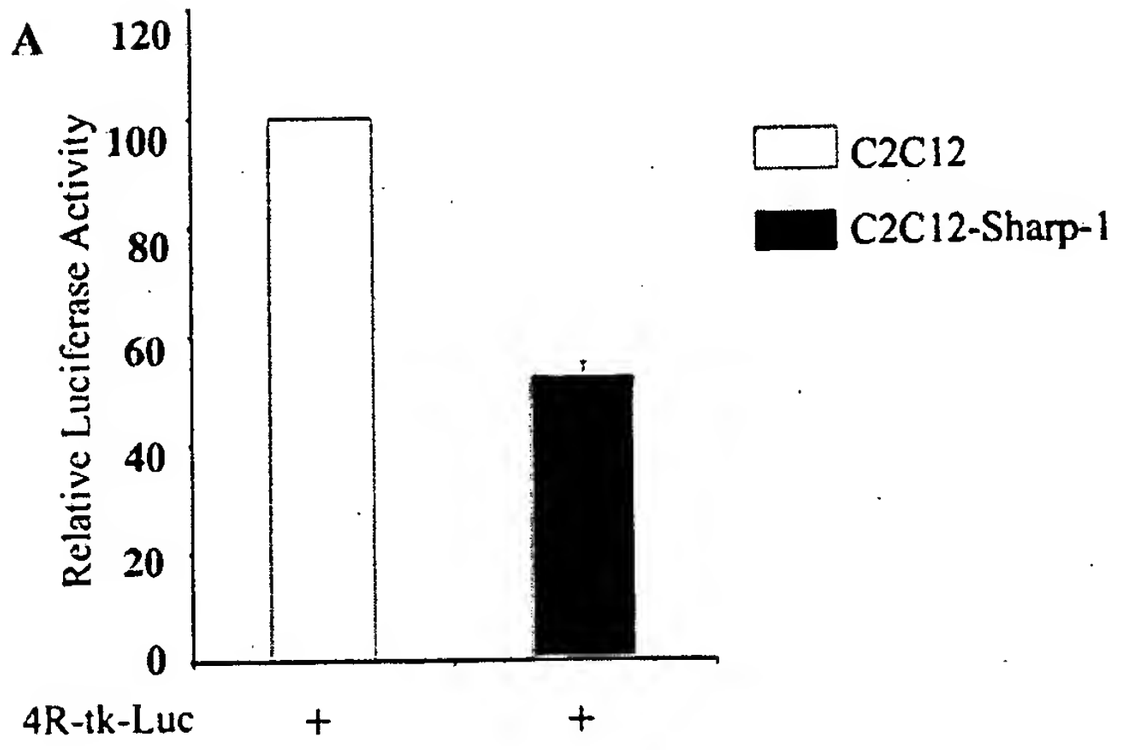
FIG. 4. **C2C12-Sharp-1 myoblasts exit the cell cycle earlier than control myoblasts.** *A*, C2C12 and C2C12-Sharp-1 cell lines were grown in GM or DM for 24 or 48 h as indicated. To identify cells in S-phase, BrdUrd was added to GM or DM for 2 h. Cells were fixed and immunostained with anti-BrdUrd antibodies. *B*, expression of Sharp-1 in myoblasts affects cell cycle markers. C2C12 and C2C12-Sharp-1 cell lines were grown in GM to ~90% confluence and transferred to DM for 24 and 48 h. Total proteins were extracted and analyzed by Western blotting. Membranes were hybridized with anti-cyclin D1 and anti-p21 antibody. EF1α served as an internal control for loading of proteins in each lane.

MyoD activity by protein-protein interactions with MyoD and E47. We therefore tested whether the inhibition of myogenic differentiation by Sharp-1 could be rescued by expression of MyoD alone or the tethered MyoD~E47 heterodimer. C2C12-Sharp-1 clones were transiently transfected with expression vectors for MyoD, MyoD~E47, or vector alone. As a control, C2C12 cells were transfected with vector alone. Transfected C2C12-Sharp-1 cells were transferred to differentiation medium for 3 days and analyzed for MHC expression by immunostaining (Fig. 8*A*), and the extent of differentiation was quantified by counting the number of multinucleated myotubes (Fig. 8*B*) in four different fields. The extent of differentiation was compared with vector-transfected C2C12 cells, which was given a value of 100%. The expression of ectopic MyoD and MyoD~E47 was determined by Western blot analysis (Fig. 8*C*).

After 72 h of incubation in differentiation media, vector-transfected C2C12 cells showed high levels of MHC expression and differentiation (100%), whereas C2C12-Sharp-1 cells diferentiated poorly (1%). The expression of exogenous MyoD alone partially rescued MHC expression and differentiation in C2C12-Sharp-1 cells to 20%, whereas expression of equivalent levels of MyoD~E47 restored differentiation and MHC expression to 70% of that seen in control C2C12 cells. The extent of rescue with MyoD and MyoD~E47 is consistent with the degree of transcriptional inhibition of these proteins (Fig. 7, *A* and *B*) and indicate that dimerization with MyoD or E-proteins does plays a significant role in Sharp-1-mediated inhibition of myogenesis. Nevertheless, formation of inactive heterodimers does not appear to be the only mechanism, because expression of MyoD~E47 was not sufficient to fully rescue the differentiation defect consistent with the ability of Sharp-1 to also inhibit MyoD~E47 activity. Taken together these data support a model where Sharp-1-mediated inhibition of myogenic bHLH activity occurs at multiple levels that include interactions with MyoD, E-proteins, as well as with MyoD~E47 heterodimers.

DISCUSSION

Members of the bHLH family of transcription factors have been shown to play critical roles in cellular differentiation, growth, and apoptosis (47). Sharp-1/Dec2 was originally identified in rat brain (36) and subsequently in human and mouse as well (33, 40). We and others have shown that Sharp-1 is expressed in a number of tissues (33, 39, 40); however, its role



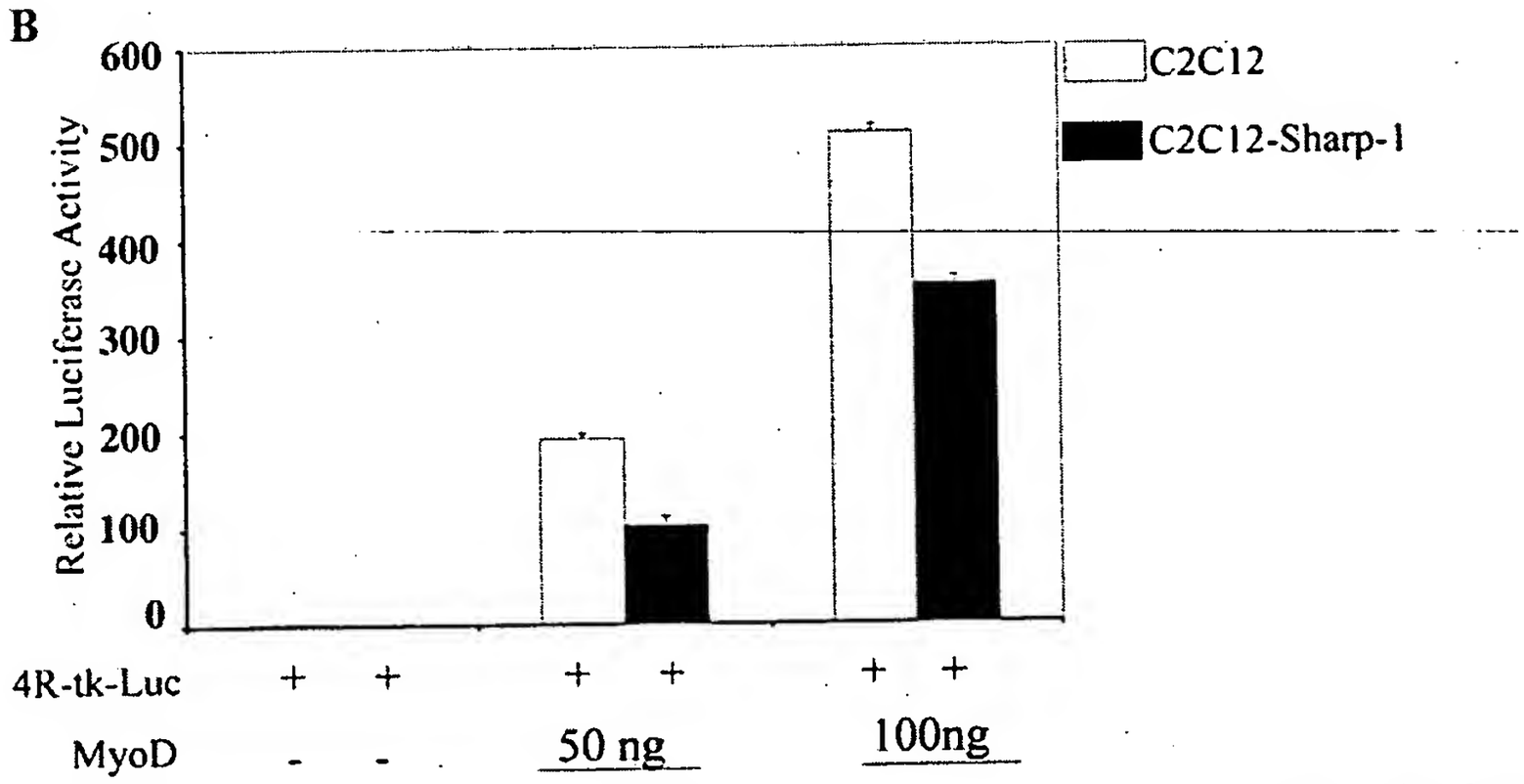
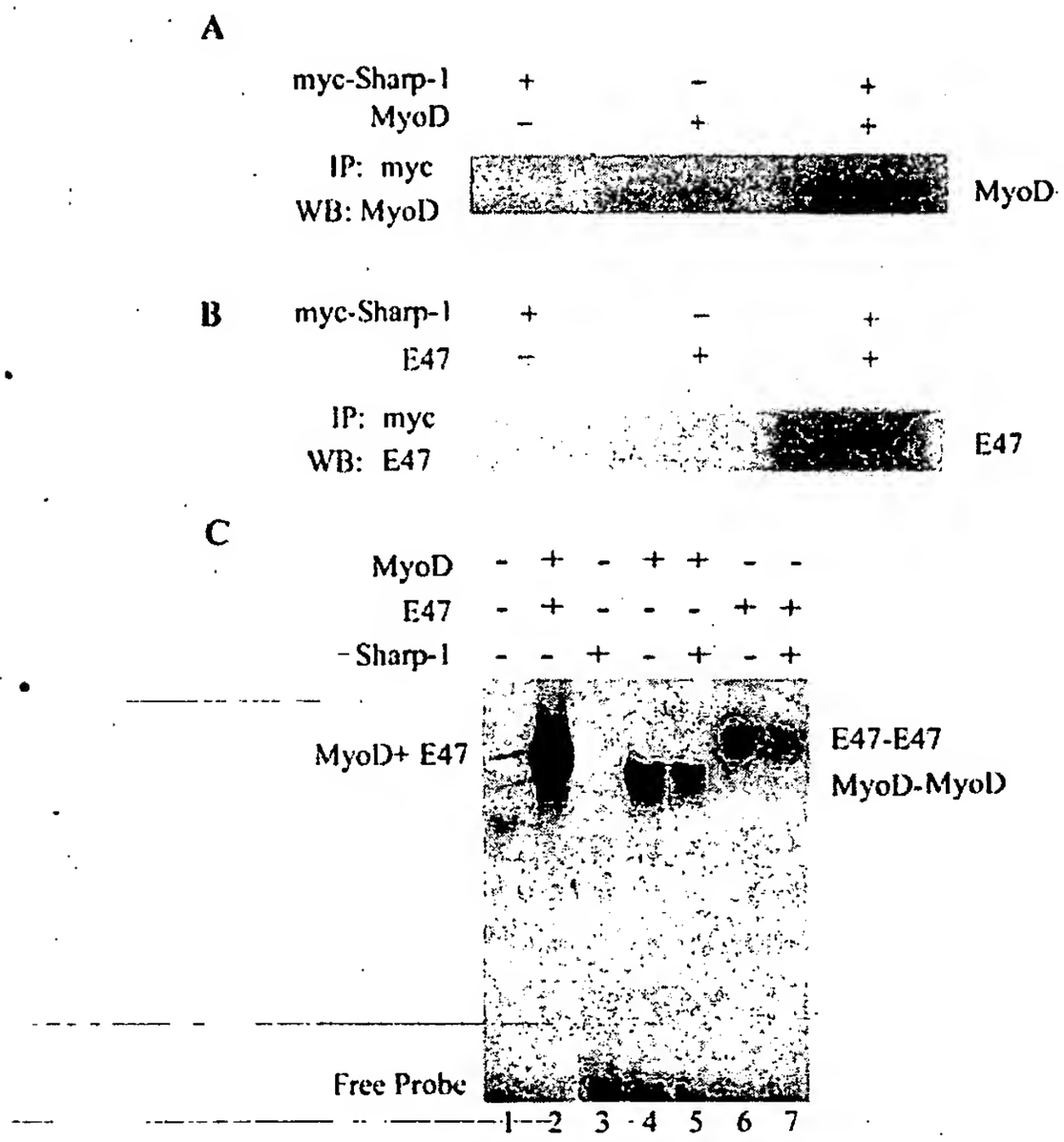


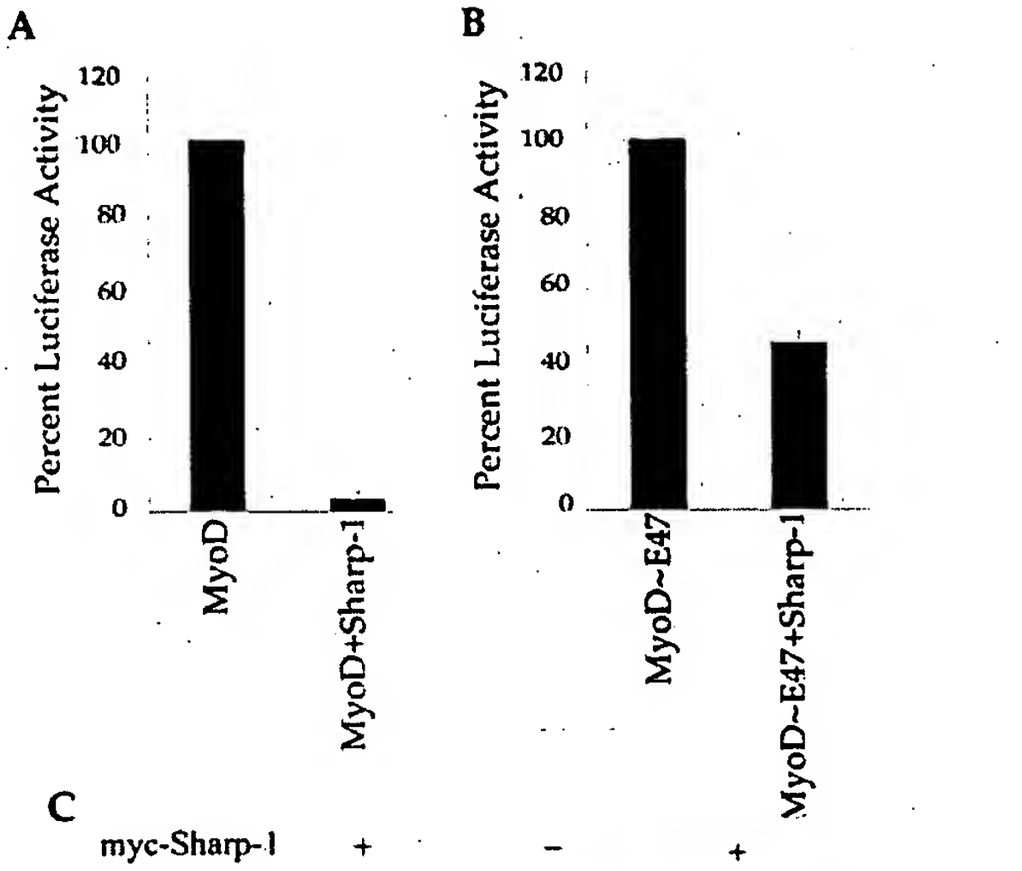
FIG. 5. **Sharp-1 inhibits MyoD activity.** *A*, C2C12 and C2C12-Sharp-1 cells were transfected with 50 ng of the E-box-dependent reporter construct 4R-tk-luc, and the basal activity of the reporter was measured after 48 h. *B*, expression of MyoD alone is not sufficient to overcome Sharp-1-mediated inhibition of E-box activity. C2C12 and C2C12-Sharp-1 cells were transfected with 50 ng of 4R-tk-luc reporter with increasing amounts (50 and 100 ng) of a MyoD expression vector. Luciferase activity was measured 48 h after transfection. A representative experiment is shown; similar results were obtained in replicate experiments.

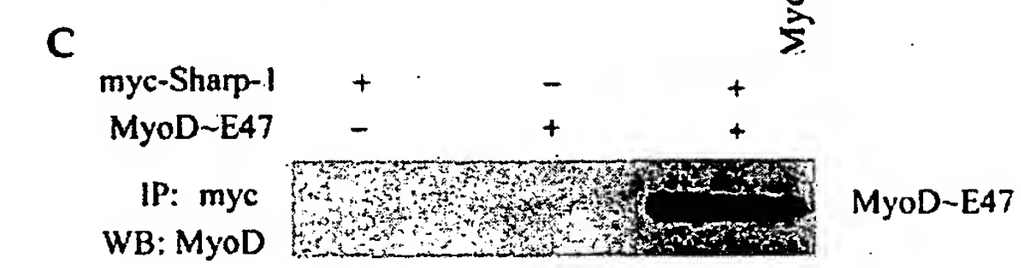
in regulation of cellular differentiation or any other biological process has not been demonstrated so far. In this report, we provide evidence that Sharp-1 is a negative regulator of muscle differentiation. Consistent with an inhibitory role, Sharp-1 is expressed at higher levels in proliferating myoblasts, and its expression is down-regulated during differentiation. Furthermore, overexpression of Sharp-1 in C2C12 myoblasts results in inhibition of muscle differentiation by negatively regulating MyoD activity.

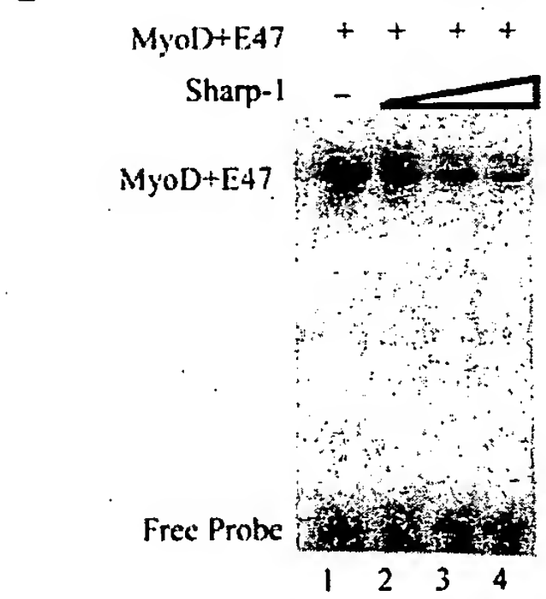
Sharp-1 Promotes Cell Cycle Exit but Inhibits Terminal Differentiation of Muscle Cells—Differentiation of skeletal muscle can be divided into two major steps: 1) withdrawal of myoblasts from the cell cycle and 2) expression of muscle differentiation genes. To determine the mechanism by which Sharp-1 inhibits myogenesis, we analyzed markers for cell cycle progression as well as for terminal differentiation. The effects on cell cycle progression were examined by analysis of cyclin D1 expression as well as by immunostaining with anti-BrdUrd antibodies. Interestingly, cyclin D1 expression, which is a marker of proliferating cells, was expressed at a lower level in C2C12-Sharp-1 cells in GM and declined to almost undetectable levels within 24 h of induction of differentiation. Consistent with these observations, Sharp-1-overexpressing cells had significantly lesser number of $BrdUrd^+$ cells both in GM and in DM. Thus, the differentiation block induced by Sharp-1 is not due to defects in cell cycle exit. Previous studies have shown that forced expression of cyclin D1 induces myoblast proliferation and inhibits differentiation (18). Because cyclin D1 levels are reduced in C2C12-Sharp-1 clones even in GM, it is likely that inhibition of cyclin D1 expression is one mechanism by which Sharp-1 causes growth suppression. These studies indicate that, similar to Stra13, whose overexpression results in growth suppression of several cell types (48–52), overexpression of Sharp-1 also results in growth inhibition of C2C12 cells. Unlike oncogenes that inhibit differentiation by promoting proliferation and preventing cell cycle exit (18–20), the inhibitory effects of Sharp-1 on differentiation can be uncoupled from its effects on cell cycle withdrawal. Inhibition of TGFβ signaling via expression of a truncated type II TGFβ receptor in C2C12 cells also results in growth suppression but blocks terminal differentiation (53). However, the truncated TGFβ receptor inhibits MyoD expression and thereby blocks differentiation. Thus, although the overall effects of inhibiting TGFβ type II receptor are similar to Sharp-1, the mechanisms by which they inhibit differentiation vary between these two genes.

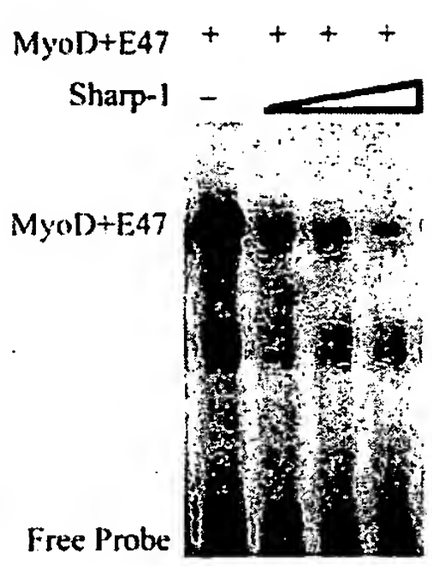
In contrast to promoting cell cycle exit, Sharp-1 potently











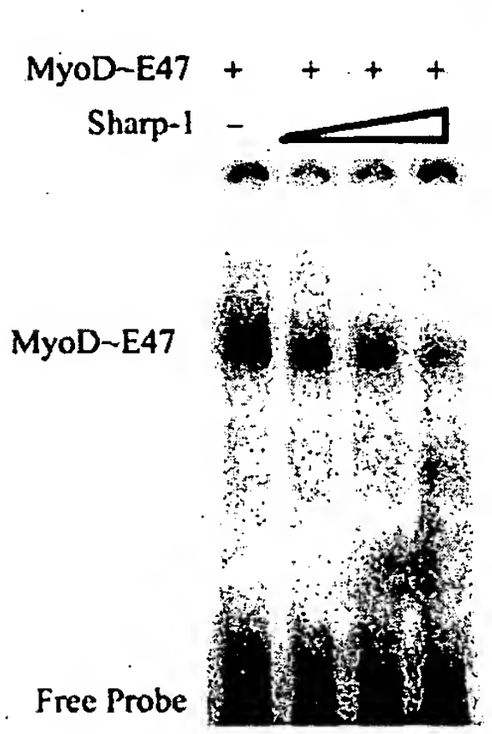


FIG. 6. **Sharp-1 interacts with MyoD and E47 and inhibits DNA binding to E-box sites.** *A*, C3H10T1/2 cells were transfected with expression vectors for myc-Sharp-1 and MyoD individually or together as indicated. 48 h after transfection, samples were immunoprecipitated with myc antibody 9E10 and analyzed for interactions on a Western blot with polyclonal MyoD antibody. *B*, C3H10T1/2 cells were transfected with expression vectors for myc-Sharp-1 and E47 as indicated. Immunoprecipitation was performed as described above, followed by immunoblotting with E47 antibody. *C*, Sharp-1 inhibits MyoD and E47 from binding DNA at an E-box site. *In vitro* translated MyoD, E47, and Sharp-1 were incubated with labeled oligonucleotides containing an E-box site from the MCK promoter. MyoD-E47 heterodimers (*lane 2*), as well as MyoD homodimers (*lane 4*) and E47 homodimers (*lane 6*) bound to E-box site, but no binding was seen with Sharp-1 homodimers (*lane 3*) or Sharp-1-E47 heterodimers. Addition of Sharp-1 to MyoD or E47 decreased binding of MyoD and E47 homodimers (*lanes 5* and *7*). *Lane 1* represents lysate alone. *D*, Sharp-1 inhibits binding of MyoD and E47 heterodimers to the MCK promoter. *In vitro* translated MyoD and E47 heterodimers formed a DNA protein complex on the MCK promoter. Increasing amounts of Sharp-1 inhibited binding of MyoD-E47 heterodimers, and no additional complexes corresponding to Sharp-1 homodimers or Sharp-1-E47 heterodimers were detected. *E*, same as *D*, using oligonucleotides corresponding to multimerized class A E-box sites.

FIG. 7. **Sharp-1 inhibits transcriptional activity of MyoD~E47 tethered construct.** *A*, C3H10T1/2 cells were transiently transfected with 4R-tk-luc (50 ng) and MyoD (50 ng) in the absence or presence of Sharp-1 (50 ng) as indicated. *B*, C3H10T1/2 cells were transfected with 4R-tk-luc (50 ng) and MyoD~E47 (50 ng) expression vector in the absence or presence of Sharp-1 (50 ng). The data shown are representative of three independent experiments performed in triplicate. Activity is expressed as the percentage of the activity obtained with 4R-tk-luc with MyoD or MyoD~E47 alone. *C*, Sharp-1 interacts with MyoD~E47 tethered construct *in vivo*. C3H10T1/2 cells were transfected with expression vectors for myc-Sharp-1 (*lane 1*), MyoD~E47 (*lane 2*), or both MyoD~E47 and myc-Sharp-1 (*lane 3*). 48 h post transfection cells were immunoprecipitated with 9E10, followed by immunoblotting with MyoD antibody. *D*, Sharp-1 inhibits binding of MyoD~E47 to E-box sites. Electrophoretic mobility shift assays were performed with radiolabeled oligonucleotides containing class A E-box sites and *in vitro* translated MyoD~E47 and Sharp-1. As expected, MyoD~E47 bound to the E-box sequence (*lane 1*). Addition of increasing amount of Sharp-1 (*lanes 2–4*) resulted in a decrease of MyoD~E47 binding.

repressed expression of genes required for differentiation such as p21, myogenin, MEF2C, and MHC. Despite the rapid cell cycle exit, p21 expression was not up-regulated in C2C12-Sharp-1 cells to levels seen in control cells. MyoD regulates the cell cycle independent of its effects on muscle differentiation, which occurs at least in part by up-regulation of the cyclin-dependent kinase inhibitor $p21^{Cip1}$ (42, 43). MyoD also regulates the expression of myogenin, which in turn is required for MEF2C expression (54–58). Thus, defective MyoD activity

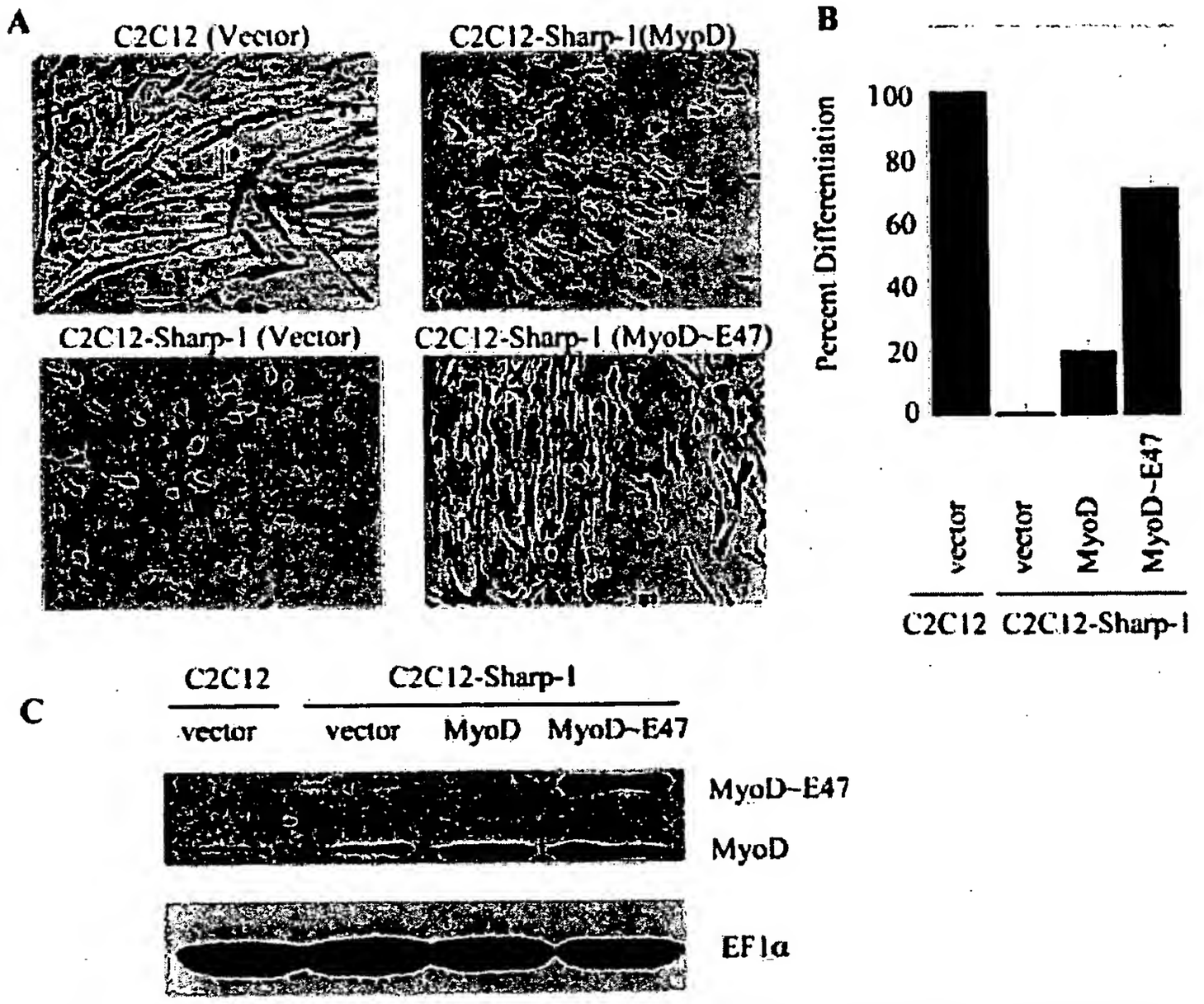


FIG. 8. **MyoD~E47 tethered construct rescues differentiation in C2C12-Sharp-1 myoblasts.** *A*, C2C12-Sharp-1 myoblasts were transfected with expression vectors for MyoD, MyoD~E47, or vector alone. Parental C2C12 cells were also transfected with empty vector as control. Cells were transferred to DM for 3 days and analyzed for expression of MHC by immunostaining. *B*, differentiation of control C2C12 cells and C2C12-Sharp-1 cells transfected with vector, MyoD, and MyoD~E47 was quantified in four fields by counting the number of multinucleated myotubes. The extent of differentiation of C2C12-Sharp-1-transfected cells was plotted relative to differentiation of vector-transfected C2C12 cells, which were given a value of 100%. *C*, expression of exogenous MyoD and MyoD~E47 in C2C12-Sharp-1 cells. Proteins were extracted from parental C2C12 cells transfected with vector alone and from C2C12-Sharp-1 cells transfected with vector alone, MyoD, or MyoD~E47 tethered construct after culturing in DM for 3 days and then analyzed by Western blotting for MyoD expression. EF1α served as a control for the loading of proteins in each lane.

likely accounts for reduced p21 and myogenin expression in C2C12-Sharp-1 cells. Because myogenin is critical for differentiation of skeletal muscle (16, 17), its impaired induction may result in reduced MEF2C expression, and the inhibition of terminal differentiation in C2C12-Sharp-1 cells.

Sharp-1 Inhibits Myogenic bHLH Transcription Factor Activity by Protein-Protein Interactions—The biochemical and molecular mechanisms by which Sharp-1 inhibits MyoD activity and myogenic differentiation are complex and occur at multiple levels. Because Sharp-1 interacts with MyoD and E47 (Ref. 33, and this study), the formation of MyoD-Sharp-1 or E47-Sharp-1 heterodimers could result in a decrease in the net availability of MyoD and E47 resulting in an inhibition of MyoD activity. In support of this possibility, Sharp-1 inhibited DNA binding of MyoD and E47 homodimers as well as MyoD+E47 heterodimers on E-box sites in gel shift experiments. Moreover, the difference in the relative extent of repression of MyoD (90%) *versus* MyoD~E47 (50%) activity suggests that formation of heterodimer complexes with bHLH factors does indeed account for some of the repressive effects of Sharp-1. Similar to Sharp-1, Id interacts with MyoD and E-proteins and inhibits myogenic differentiation. Id functions primarily by sequestration of E-proteins (28), and thus MyoD~E47 heterodimers (45) are resistant to inhibition by Id. In contrast, Sharp-1 represses MyoD activity even in the presence of excess E12 (33) and also inhibits transcriptional activity of MyoD~E47 heterodimers. Thus, Id and Sharp-1 differ in the mechanisms by which they repress muscle differentiation, and sequestration of E-proteins does not appear to be the primary mechanism by which Sharp-1 inhibits myogenic bHLH activity. Sharp-1 homodimers and Sharp-1-E47 heterodimers did not exhibit any detectable binding to the E-box sites that we tested in this study. The physical association of Sharp-1 with MyoD, E-proteins, and MyoD~E47 heterodimers, as well as the absence of detectable Sharp-1 homodimer or Sharp-1-E47 heterodimer binding at E-box sites, suggests that protein-protein interactions rather than DNA binding plays a significant role in the inhibition of MyoD activity by Sharp-1. In this regard, the effect of Sharp-1 is similar to Hes6, which also appears to inhibit myogenic differentiation through protein-protein interactions rather than direct DNA binding (59).

In addition to interacting with MyoD and E47, Sharp-1 inhibits transcriptional activity of the tethered MyoD~E47 heterodimer, albeit to a lesser extent than MyoD. The effect of Sharp-1 on MyoD~E47 transcriptional activity, though surprising, is not unprecedented. Similar to Sharp-1, both Mist and MyoR also repress transcriptional activity induced by MyoD~E47 heterodimers (30, 31), although the mechanisms underlying this repression have not been defined. In addition, the muscle LIM protein has been shown to interact with MyoD~E47 heterodimer enhancing its DNA binding activity (60). In contrast to the muscle LIM protein, increasing amounts of Sharp-1 led to an effective decrease in MyoD~E47 DNA binding. Although the domains through which Sharp-1 interacts with MyoD~E47 remain to be investigated, the inhibition of DNA binding and transcriptional activity of MyoD~E47 heterodimers add an additional step of regulatory control via which Sharp-1 exerts its inhibitory effects on myogenic bHLH factors.

Consistent with the fact that Sharp-1 inhibits transcrip-

tional activity of MyoD to a greater extent than MyoD~E47, the differentiation defect in C2C12-Sharp-1 cells is rescued to a significantly higher level by expression of MyoD~E47 as compared with equivalent levels of MyoD alone. Interestingly, expression of MyoD~E47 in C2C12-Sharp-1 cells resulted in an increase in the level of endogenous MyoD, verifying that MyoD~E47 was functionally active in these cells. The increase in endogenous MyoD in MyoD~E47-expressing cells is equivalent to those transfected with MyoD alone. This increase in endogenous MyoD expression in MyoD~E47-expressing cells would therefore presumably contribute to restoring differentiation to the same extent as cells expressing MyoD alone (20%). Thus, the difference in the degree of rescue of myogenic differentiation between MyoD~E47 and MyoD indicates that dimerization with MyoD and E47 plays a significant role in Sharp-1-mediated inhibition of myogenesis. During mouse embryogenesis, Myf5 is the first skeletal muscle marker that is expressed at E8.0 followed by expression of myogenin (E8.5), MRF4 (E9.5), and MyoD (E10.5) (61). Sharp-1 is not expressed at detectable levels at E8.5 and E9.5 in somitic myotome (39), suggesting that it is unlikely to be critical in the initial steps of muscle development. However, during primary myogenesis that occurs between E10.5 and E14.5, Sharp-1 is expressed in the myotome, limb buds, and branchial arches. Because Sharp-1 inhibits MyoD-dependent E-box activity *in vitro*, it may function to regulate the expression of muscle specific genes during primary or secondary myogenesis.

While Sharp-1 is expressed in muscle and regulates myogenesis by interfering with MyoD function, its expression during mouse embryogenesis is not limited to the myogenic lineage (39). Sharp-1 is expressed at E8.5 onwards in the developing nervous system, heart, and other tissues whose differentiation is also critically dependent on bHLH factors. Thus, similar to its role in muscle cells, Sharp-1 may regulate neurogenic or cardiac differentiation during embryogenesis through its ability to interfere with function of neural and cardiac bHLH factors and E-proteins.

Taken together, our data demonstrate that Sharp-1, which belongs to the group E subfamily of bHLH factors, regulates myogenic differentiation by antagonizing the functional activity of class A myogenic bHLH factors and E-proteins via protein-protein interactions. Future studies aimed at evaluating its role *in vivo* will help elucidate more precisely the role of Sharp-1 in skeletal muscle biology.

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